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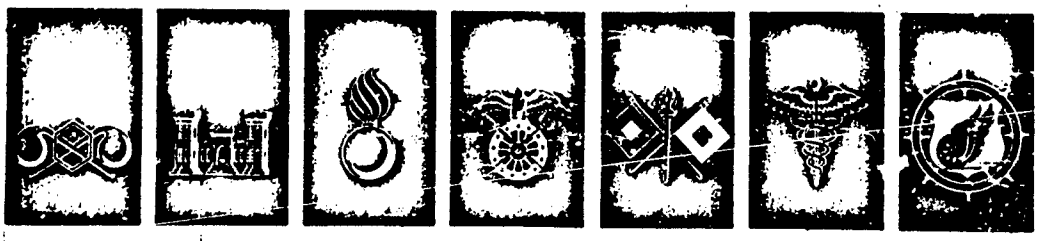

TITLE: Evidence That Rhesus Monkeys Die from Anthrax Toxin
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ABSTRACT: Evidence that a toxin produced by B. anthracis causes death in animals has been extant since as early as 1931. Recognition of these evidences and their reverification have been left until this decade, when Smith et al. (1955) and later workers demonstrated that the terminal shock from toxin is the major cause of death in anthrax. In this paper evidence is presented that: (1) Anthrax toxin can be demonstrated in body fluids of the Rhesus monkey at death. (2) Anthrax toxin is present in increasing amounts in body fluids of Rhesus monkey dying of anthrax. (3) In vitro produced, sterile anthrax toxin is capable of causing the death of Rhesus monkey. (4) Anthrax antiserum protects the Rhesus monkey against in vitro toxin produced by B. anthracis. These findings imply that the treatment of the disease in man should be revised to include use of antitoxin for neutralization effects.

TITLE: The Ecological and Epidemiological Aspects of Biological Field-Testing at Dugway Proving Ground

AUTHOR: PALMER
Dugway Proving Ground

ABSTRACT: The objectives and actual functions of the unique organization are related as regards biological field-testing safety. From simultaneous disease survey data of wildlife and livestock populations, on and near the Proving Ground, indicate that there has been no escape of any agent, but incidence is apparently due to endemic diseases of native origin.

A determination has been made from ecological studies classifying the area into several types as delineated by general relief, soil type and plant cover from which can be predicted the quantity and type of native fauna. These data have had practical application in devising a control program and for recommendations as to testing activities.

EVIDENCE THAT RHESUS MONKEYS DIE FROM ANTHRAX TOXIN

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Some of the earliest evidence of toxin production during the growth of Bacillus anthracis, although apparently unrecognized as such, were presented by Sobernheim (8) who described anthrax as producing edema and hemorrhage and interferring with blood clotting and carbohydrate metabolism. Later, Howie and Cruickshank (3) showed that they could increase the lethal effect of anthrax by injecting shock-producing substances into animals previously challenged with B. anthracis. Smith et al. (7) observed that the terminal phase of anthrax is accompanied by secondary shock which they reported to be the major cause of death. Their work, partly confirmed by Ross (6), lead to the demonstration of a specific toxin isolated from the plasma of guinea pigs which had died of anthrax. The plasma, sterilized by filtration, was shown to produce an edema when injected into the skin and death when injected intravenously into other guinea pigs. Recently, Stanley and Smith (9) and Beall and Taylor (1) independently discovered that "toxin" is composed of at least 3 components, identified by the latter authors as protective antigen, guinea pig edema factor and rat lethal factor. They also showed that rat lethal factor is toxic only in association with protective antigen.

It is our purpose to report on work demonstrating: (1) the presence of toxin in blood and lymph of the Rhesus monkey (2)quantitative increases of toxin in blood and lymph prior to death and (3) other evidence of toxemic death.

In order to obtain body fluids for these studies workers in our laboratories (Hodges and Rhian, 2) developed a survival surgical procedure for cannulation of the thoracic and right lymph ducts and the jugular vein of the Rhesus monkey. The procedure involves opening the chest to enter the superior mediastinum by bisecting the sternum thus exposing for cannulation both right and thoracic lymph

ducts. These ducts are pictured diagrammatically in (Fig. 1). Simultaneously a venous cannula is placed in the heart by jugular vein cannulation. Following this procedure the monkeys recover rapidly as shown in this picture of a monkey 24 hours post surgery. (Fig. 2) Lymph is collected continuously and blood samples can be drawn as desired. For this study, changes in concentration of microorganisms and toxin in the lymph and blood were measured following challenge with B. anthracis.

Quantitative assay for toxin was based on two procedures. The first, that of Beall and Taylor (1) based on the susceptibility of Fischer rats to anthrax toxin, and the second, based on the agar diffusion technique described by Thorne and Belton (10) to measure the precipitation of antibody and antigen.

Our studies are based on the following observations: (1) toxin is present in the terminal blood of monkeys dying of anthrax, (2) toxin increases in concentration in both the lymph and blood reaching a maximum at death, (3) sterile toxin alone causes death of the monkey and (4) rapid death occurs in monkeys following an injection of 10^{11} B. anthracis spores.

During the course of work reported elsewhere, in which the course of anthrax and the development of septicemia in the Rhesus monkey was studied, we tested the terminal blood of 15 Rhesus monkeys for toxin. In 12 of these terminal blood samples toxin was demonstrated by death of the rat.

Increasing concentrations of toxin, to a maximum at death, were obtained from two monkeys studied to date. The thoracic lymph duct and the jugular vein of the first monkey were cannulated. Twenty four hours post-surgery the monkey was inoculated intradermally with 10^5 virulent anthrax spores. The site of inoculation drained to the popliteal lymph node. The animal died 41.5 hours after challenge. Lymph was collected regularly during that time and the samples were kept separated so that changes in the lymph could be correlated with time. Changes in organisms and toxin levels are shown in Table 1. The results of three assays of each sample are shown. For instance, the lymph collected from this monkey at 17 hours prior to death was found to contain $< 0.2 \times 10^6$ orgs/ml while lymph collected 3 hours prior to death contained 88×10^6 orgs/ml. During this same period the toxin titer increased from 0 to a 1/5 dilution. When 1 ml of the 3-hour lymph sample was injected intravenously into each of two rats they survived for an average time of 144 minutes. From this table it can be seen that the lymph collected near the death of the monkey was both more antigenic and more lethal to rats than lymph collected several hours prior to death. This observed increase in toxin is statistically significant ($P < .001$).

Sterile lymph collected before challenge was not toxic to rats. It was also proven that a combination of two parts of the toxic lymph and one part of anthrax antiserum* was non-toxic. The above tests demonstrate that death was caused by toxin produced by B. anthracis infection.

The second experiment was similar to the first except the right lymph duct, which drains a major portion of the lungs, was used instead of the thoracic duct. Also, the monkey was challenged by the aerosol route with 3×10^5 anthrax spores. The data from this monkey, which died in 57 hours, are shown in Table 2. Since the lymph flow was very slow only a small amount could be collected over any time period. Thus the toxin test used only one rat per point and challenge was with only 0.5 ml of lymph. Therefore, the times to death on this table do not compare directly to those on the preceding table. The regression of time prior to death of the monkey on time to death of the rat has a slight significant slope ($P < .10$). Although the level of significance is low, probably due to the small number of rats, there is an increase in toxin concentration during the period of observation. These observations parallel those made in the previous experiment. The agar precipitation test was not performed on any of these samples because of insufficient material.

The terminal blood of each of these two monkeys was assayed for toxin. That of the first monkey was found to be negative whereas the terminal blood of the second monkey was toxic for rats. From Fig. 3 it is seen by the dotten line that the concentration of organisms in the lymph of the first monkey built up rapidly to approximately 10^8 organisms per ml. There was no such buildup however in the blood (solid line) of this animal. It is shown in Fig. 4 that the concentration of organisms in both the blood and lymph of the second monkey increased to relatively high levels. As with the first monkey, however, the buildup of organisms in the lymph was greater than in the blood. It was also found that the terminal blood of the second monkey was toxic for rats which died four hours after being injected with 0.5 ml of the blood.

The fact that blood of the first monkey did not develop toxin to a demonstrable level is not unexpected. We have shown that the principal extravascular route of anthrax organisms and toxin into the blood is through the thoracic and right lymph ducts. Malek et al. (4) demonstrated in sheep that at the peak of inflammation of the lymph nodes a pathological secondary lympho-venous communication is established which allows direct passage of organisms into the blood.

* Anthrax spore antiserum from hyperimmunized horse (DH-1-4A)

Since we collected all thoracic and right lymph before it entered the blood stream, we may have removed most of the organisms and toxin not actually diffused by this secondary pathological communication. Anatomically there are fewer lymph nodes in the popliteal and inguinal region than in the region draining the lungs. Hence, it is a question of the number of pathological secondary lympho-venous communications between the two lymph systems that may account for the absence of toxin and low concentration of organisms in the intradermally challenged monkey.

The data show that toxicity is proportional to the concentration of organisms in the body fluids. Specifically the lymph of both monkeys showed parallel increases of both organisms and toxicity. The blood of the first showed a negligible increase of organisms and no toxicity, whereas the blood of the second showed a substantial increase in organisms accompanied by a demonstrable toxicity. This is the first demonstration for such a build-up of toxin in body fluids of monkeys and complements the British observation on toxin in terminal blood of guinea pigs.

The next step was to show that anthrax toxin alone is sufficient to cause the death of these animals. Sterile toxin was produced in vitro by the method of Thorne et al. (11) of a potency such that 1 ml doses killed rats in an average of 80 minutes. A dose of 250 ml of this toxin was administered intracardially to a Rhesus monkey weighing 16 pounds. The monkey received the toxin with no apparent ill effects. It died 21 hours post-challenge with terminal symptoms typically associated with toxic death in rats. (i.e., partial paralysis of upper extremities and extreme respiratory distress, accompanied by massive pulmonary edema).

In another trial three 8-pound Rhesus monkeys were challenged intravenously with 200 ml, 250 ml and 400 ml respectively, of sterile in vitro produced anthrax toxin. A fourth monkey received 250 ml of toxin followed by three 50 ml injections of antiserum given 0, 1/2 and 21 hours post-challenge. The animal receiving 200 ml survived; the animals challenged with 250 ml and 400 ml died in 60 and 33 hours respectively. The animal receiving anthrax antiserum survived. These data indicate a dose-response relationship between anthrax toxin and death of the Rhesus monkey and that toxin was neutralized by antiserum.

The final evidence of toxemic death of Rhesus monkeys was revealed when a dose response curve for challenge doses of 10^5 through 10^{11} spores was run. The expected dose response was observed for the doses 10^5 through 10^{10} spores with death occurring between 20 and 50 hours. Three monkeys given 10^{11} spores, however, died 2 hours after challenge with symptoms of toxemic death. We interpret these observations as showing that 10^{11} spores, on germination and outgrowth,

produce enough toxin to kill a monkey. The entirely different course of the disease as evidenced by short time to death and only symptoms of toxemia indicate that these three monkeys died of anthrax toxin.

In summary it has been shown that: (1) Anthrax toxin can be demonstrated in body fluids of the Rhesus monkey at death. (2) Anthrax toxin is present in increasing amounts in body fluids of the Rhesus monkey dying of anthrax. (3) In vitro produced, sterile anthrax toxin is capable of causing the death of Rhesus monkeys. (4) In the one instance in which it was tried, anthrax antiserum protected the Rhesus monkey against in vitro toxin produced by B. anthracis.

The importance of toxin in affecting the pathogenic course and therefore the treatment of the disease in man remains to be determined. It would seem probable that the effect of toxin in man is like that in other animals. This additional knowledge of toxin has a significant implication on the treatment of the disease. Since the introduction of antibiotics, all treatment is directed against the organism. It seems clear now that in order to be fully effective in treating systemic anthrax it will be necessary also to direct treatment against the toxin. Thus antibiotics alone do not constitute adequate treatment, except, of course, when begun in a very early stage of the disease. It now remains to develop an effective antitoxin since the laboratory and clinical methods available to us at this time are inadequate for early detection of the disease. Like conclusions were drawn by Plotkin et al. (5) following their observations on the New Hampshire epidemic of inhalation anthrax who state "In view of the importance of toxemia to the outcome of the experimental disease, the use of antitoxin seems logically indicated in human inhalation anthrax".

REFERENCES

1. Beall, F.A. and M.J. Taylor. 1962 Accepted by J. Bact. for publication.

2. Hodges, D.R. and M.A.Rhian. 1962 J. Expr. Med and Surgery, 20th Vol.

3. Howie, J.W. and J. Cruickshank. 1947 J. Path. and Bacteriol. 59: 127.

4. Malek, P., J. Kole and F. Zak. 1959 J. of Bact., Parasit. and Hyg.; Abt. I Orig. 174: 94.

5. Plotkin, S.H., P. S. Brachman and M. Utell. 1960 The Amer. Jour. of Med. Vol. XXIX: 992.

6. Ross, J.M. 1955 Brit. J. Expr. Path. 36: 336.

7. Smith, H., J. Keppie, J.T. Stanley and P.W. Harris-Smith. 1955 J. Gen. Microbiol. 17: 731.

8. Sobernheim, G. 1931 Handbuck de Pathogenen Mikro-organismen 3: 1041 Gustov Fischer, Jena.

9. Stanley, J.L. and H. Smith. 1961 J. Gen. Microbiol. 26: 49.

10. Thorne, C.B. and F.C. Belton. 1957 J. Gen. Microbiol. 17: 505.

11. Thorne, C.B., D.M. Molnar and R.E. Strange. 1960 J. Bact. 79: 450.

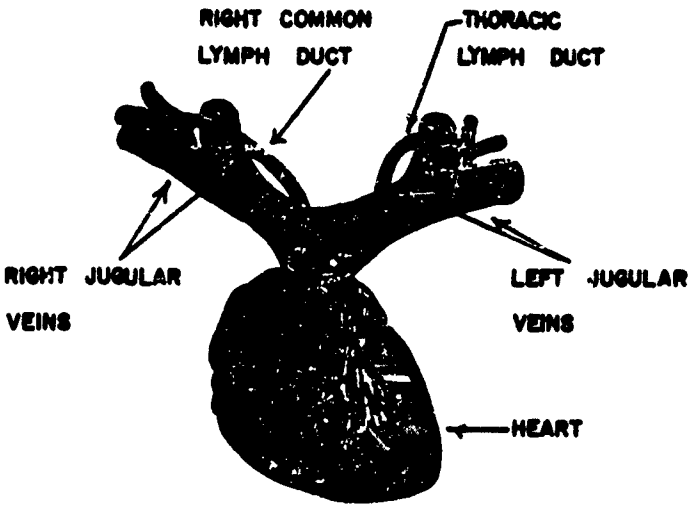

FIGURE 1. LOCATION OF THORACIC AND COMMON LYMPH DUCTS ANTERIOR VIEW.

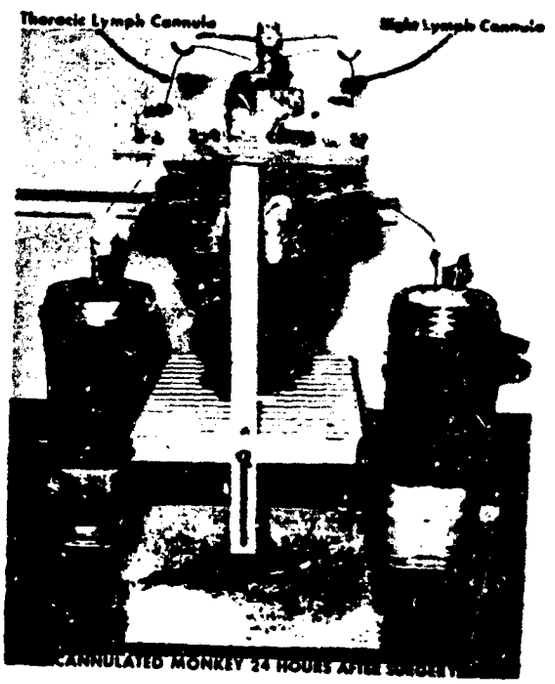

FIGURE 2.

TIME PRIOR TO DEATH OF MONKEY (HRS.)	ORGS. IN LYMPH (10^6/ML)	TESTS FOR TOXIN	
		RAT (MIN. TO DEATH)	PRECIPITIN (TITER)
17	<.2	S	0
13	52	S	1/3
11	71	360	1/4
9	69	233	1/4+
7	168	143	1/5
5	85	126	*
3	88	144	1/5
DEATH	69	76	*

* NOT ENOUGH LYMPH COLLECTED TO RUN TITER.

S SURVIVED.

TABLE I. ANTHRAX ORGANISMS AND TOXIN IN LYMPH FROM MONKEY CHALLENGED BY INTRADERMAL ROUTE.

TIME PRIOR TO DEATH OF MONKEY (HRS.)	ORGS. IN LYMPH	TEST FOR TOXIN
		RAT
	10^6/ ML	(MIN. TO DEATH)
8	15	S
7	18	240
5	19	237
3	15	180
DEATH	26	177

S SURVIVED

TABLE 2. ANTHRAX ORGANISMS AND TOXIN IN LYMPH FROM MONKEY CHALLENGED BY AEROSOL ROUTE.

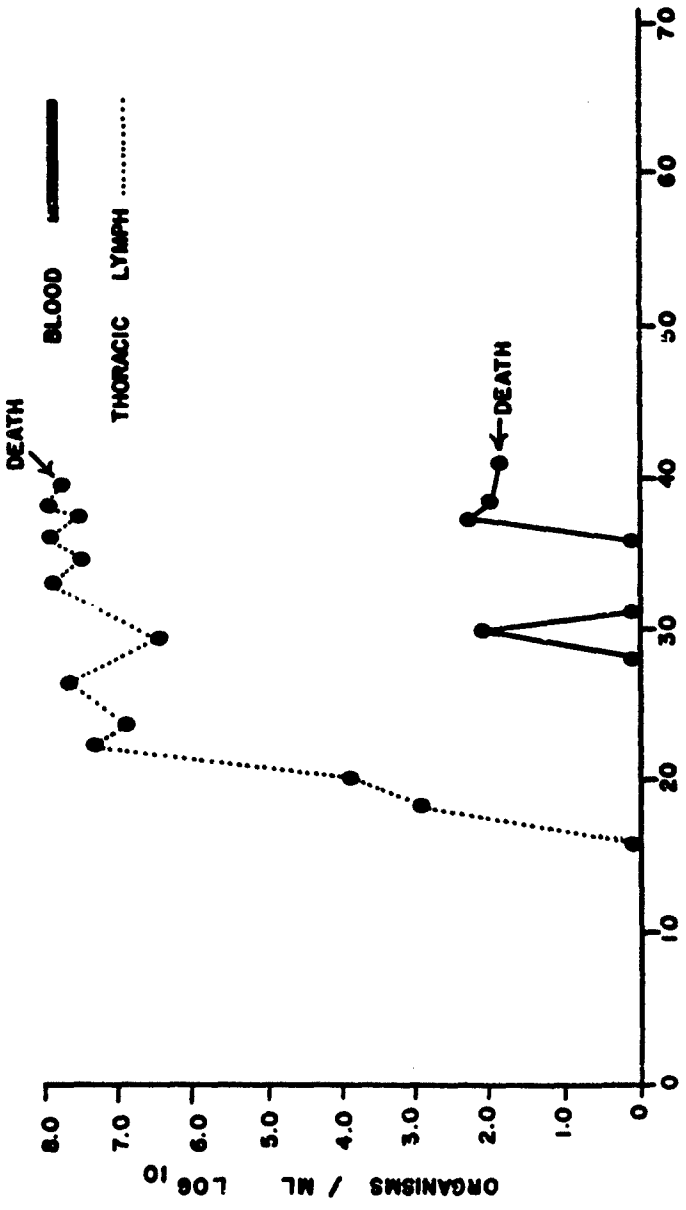

FIGURE 3. BACILLI IN THE BLOOD AND THORACIC LYMPH OF A MONKEY INTRADERMALLY CHALLENGED WITH SPORES OF P. ANTHRACIS.

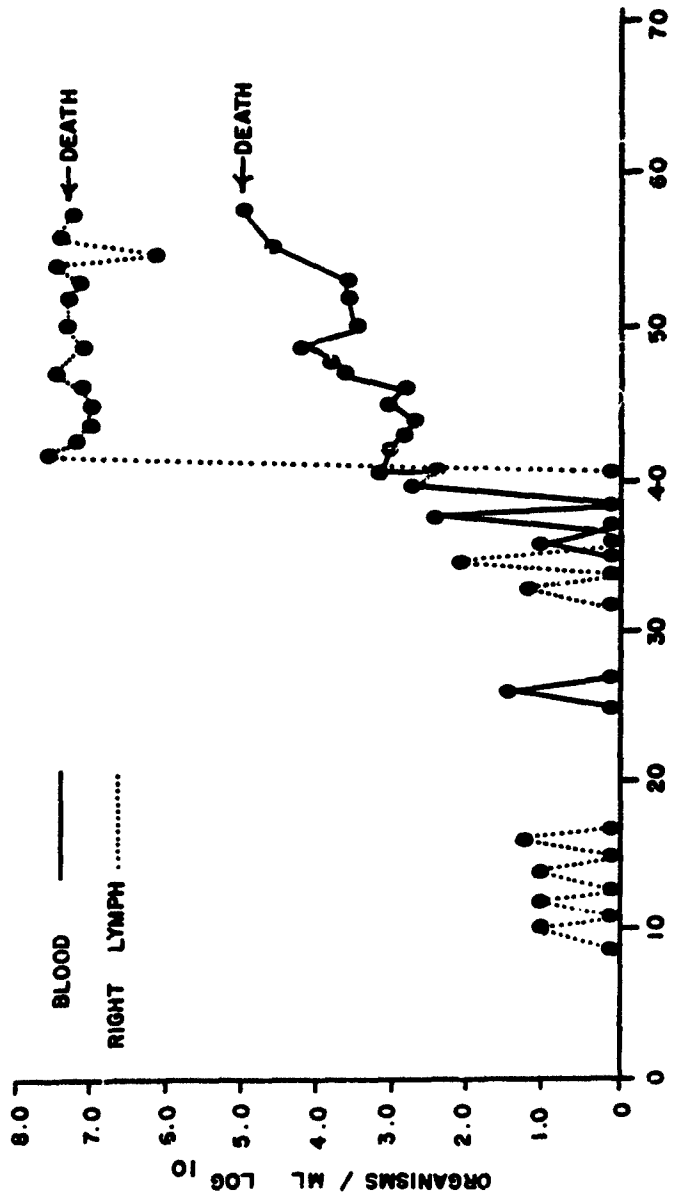

FIGURE 4. BACILLI IN THE BLOOD AND RIGHT LYMPH OF A MONKEY FOLLOWING AEROSOL CHALLENGE WITH SPORES OF B. ANTHRACIS.